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NUTRITIVE VALUE OF THE WOOD-ROTTING FUNGI AND THEIR SYNTHETIC PRODUCTS

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GENERAL AND SPECIFIC OBJECTIVES

General Objective

This research has as its broad objective a study of the nutritive value of the wood-rotting fungi and their products of synthesis, with a view to using these organisms or products as animal feed supplements or as sources of food materials for other purposes. Because of their marked synthetic powers these organisms may be valuable sources of protein, fats, vitamins, etc.

Specific Objectives

More specifically, this investigation involves first a systematic exploratory study of the composition of (1) the fungal mycelium, and (2) the culture fluid, as regards nitrogen compounds, fats, vitamins, etc. synthesized by the wood-rotting fungi in submerged culture. Products of fermentation may also be involved. Included in the orientation work will be representative species of these organisms, and a variety of culture conditions and nutrients. The direction of later studies--for example, increasing the yield of a specific product--will depend upon results obtained in the exploratory studies.

BASIC PROCEDURES

A. General culture method.

The procedure followed in growing the organisms is that of aerated, liquid culture (submerged culture), which method we developed for the wood rots in connection with our previous studies of the organisms. Shake cultures and/or large flask cultures with forced aeration are used, incubated at 28°C. By this means these ordinarily slow-growing fungi can be grown relatively rapidly and in large quantities in test nutrients. (For example, with this method the maximum amount of growth is obtained in about two weeks even in synthetic media, and this amount may be as much as 1 gram of dry weight of mycelium per 100 ml. of medium, depending upon the

organism and medium). Only mycelial growth in the form of pellets is produced, not spore structures. The organisms will grow in a variety of cheap, non-synthetic media such as malt extract, corn steep liquor, soy bean meal, cellulosic wastes, etc., as well as in purely synthetic culture mediums.

B. Quantity production of the organisms.

To obtain large amounts of fungal mycelium for comparative analyses at one time, the organism is grown in quantity in the test nutrients, then the mycelium is lyophilized and stored.

The stock cultures are carried on potato dextrose agar (PDA) slants, then grown in shake culture in a standard 2% malt extract (Difco, desiccated) solution to produce pellets of mycelium for inoculating the test nutrients.

To start the liquid culture, small bits of mycelium are transferred from the stock slants into 70 ml. of 2% malt extract in 250-ml. Erlenmeyer flasks. These flasks are incubated at 28°C. on a reciprocating shaking machine (stroke 1½ inches; 110 3-inch cycles per minute) for 7 days. The contents of the flask are then blended in a sterile Waring blendor for 1 minute, and an aliquot transferred aseptically to a sterile 15 ml. centrifuge tube. The mycelial suspension is centrifuged for 2 minutes at 2000 r.p.m. and washed with sterile distilled water. Centrifuging and washing are repeated twice more to minimize the carry-over of nutrients from the original malt extract to the particular test nutrient being used.

For the standard inoculum, the washed suspension of mycelial fragments is adjusted to 2.5% concentration by volume, and 0.2 ml. aliquots of this standard are used to inoculate 250-ml. flasks of the test nutrients. The organism is carried through two successive serial subcultures in each test nutrient, each of these shake cultures being treated like the malt extract culture above. The third serial subculture is prepared by adding 10 ml. of standard inoculum from the second subculture to 5 liters of test nutrient in 2½-gallon bottles. These cultures are

grown at 28°C. for from 1 to 2 weeks, depending upon the organism and its rate of growth, sterile air being forced through at a rate of about 2 liters per minute. The pellets of growth are filtered on cheesecloth and washed several times with distilled water. The mycelium is then lyophilized and stored.

C. Basal synthetic medium

Basal Synthetic Medium

Glucose	10.0 gm./liter
KH_2PO_4 (K=430, P=342 mg./l.)	1.5 gm./liter
$MgSO_4.7H_2O$ (Mg=49.5 mg./l.)	0.5 gm./liter
Thiamine monohydrochloride	1.00 mg./liter
Nitrogen source	120.0 mg./liter N
Trace elements	
B (as H_3BO_3)	0.10 mg./liter
Mn (as $MnCl_2.4H_2O$)	0.01 " "
Zn (as $ZnSO_4.7H_2O$)	0.07 " "
Cu (as $CuSO_4.5H_2O$)	0.01 " "
Mo (as $(NH_4)_6Mo_7O_{24}.4H_2O$)	0.01 " "
Fe (as $FeSO_4.7H_2O$)	0.05 " "

D. Methods for total nitrogen, protein and ash.

A micro-Kjeldahl method was used for total nitrogen, the procedure being that adopted by the Association of Official Agricultural Chemists.[1] The distillation apparatus was built according to the specifications formulated by the Committee for the Standardization of Microchemical apparatus, of the American Chemical Society.[2]

For total nitrogen, approximately 25 mg. of lyophilized, oven-dry (105°C for 24 hours) mycelium were weighed out in a tared cup fashioned from cigarette paper and place in a micro-Kjeldahl digestion flask containing 1.30 g. (± .05 g.) of potassium sulfate and 40 mg. (± 5 mg.) of mercuric oxide. Two ml. of concentrated sulfuric acid and boiling chips small enough to pass through a No. 10 sieve were added and the sample digested for 5 hours at a vigorous boil so that the acid condensed well up into the neck of the flask.

The mixture was allowed to cool and approximately 5 ml. of water were added to dissolve any solids which might have formed. The digest, including the boiling chips, was transferred to the distillation apparatus, 8 ml. of standard sodium hydroxide-sodium thiosulfate solution were added and the ammonia distilled over. Approximately 15 ml. of the steam distillate were collected in 5 ml. of 4% boric acid solution containing 4 drops of methyl red-bromcresol green indicator The distillate was diluted to 50 ml. with distilled water and the ammonia titrated with 0.01 N hydrochloric acid, using the first appearance of a red color as the end point. A blank containing everything except the sample was run in the same manner.

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[1]Official Methods of Analysis of the Association of Official Agricultural Chemists. 1950. Association of Official Agricultural Chemists, Washington, D.C.

[2]Analytical Chemistry, 23, 523, 1951.

The percentage of nitrogen in the sample was calculated using the following formula[3]:

$$\frac{\text{(ml. HCl determined - ml. in blank) X normality of HCl X equiv. wt. nitrogen X 100}}{\text{wt. sample (mg.)}} = \% \text{ nitrogen}$$

The percentage of protein was calculated as % N X 6.25.

For ash determinations, samples of about 0.4 g. of oven-dry mycelium were ignited in a muffle furnace at 600°C. for 4 hours. The residue was cooled and dried over calcium chloride in a desiccator, and weighed.

E. Methods for amino acids

These were detected and identified in mycelial hydrolysates by the method of Consden, Gordon and Martin[4], using ascending, two-dimensional paper chromatography.

Preparation of sample (acid hydrolysis)-- 50 mg. of lyophilized, oven-dry (105°C. for 24 hours) mycelium were hydrolyzed under reflux with 10 ml. of 6 N HCl for 20 hours. The excess HCl was removed by evaporation to dryness on a steam bath and the resulting residue in the beaker placed in a vacuum desiccator over soda lime for 24 hours. The hydrolysate is then taken up with about 100 ml. of water, filtered, again evaporated to dryness and finally taken up in 5 ml. of 75% ethanol.

Preparation of sample (alkaline hydrolysis - for tryptophane only)-- 50 mg. of lyophilized, oven-dry mycelium were boiled under reflux with 10 ml. of 14% barium hydroxide in an oil bath at 100°C. for 22 hours. The barium is removed with a slight excess of 1 N sulfuric acid and the barium sulfate precipitate is thoroughly washed with hot water containing a drop of acetic acid. The filtrate is concentrated to a small volume in vacuo and then evaporated to dryness in a desiccator

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[3]Jacobs, M.B. The Chemical Analysis of Food and Food Products. New York, D. Van Nostrand, 1951.

[4]Biochem. J., 38, 224, 1944.

over calcium chloride. The amino acid residue is taken up in 5 ml. of 75% ethanol.

Preparation of the amino acid standards. -- The concentrations of amino acids shown in the following table are made up in 75% (v/v) ethanol; a small amount of concentrated HCl was added to dissolve the less soluble ones. The table also shows the minimum amount of each amino acid which can be detected on paper chromatograms using the ninhydrin reaction[5].

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[5]Berry, Sutton, Cain and Berry. 1951. University of Texas Publications. Biochem. Institute Studies IV. Austin, Texas.

TABLE A

AMINO ACID STANDARDS FOR PAPER CHROMATOGRAPHY

Amino acid	Standard solution- µg/µl	Minimum amount detectable- µg	µl applied
a-alanine	5 / 5	0.5	5
arginine	5 / 5	1.0	5
aspartic	2 / 5	0.5	5
cysteine	1 / 5	*	10**
cystine	1 / 5	1.0	10**
glutanic	2 / 5	0.5	10**
glycine	5 / 5	0.25	5
histidine	5 / 5	3.0	5
hydroxyproline	5 / 5	2.0	10**
isoleucine	5 / 5	*	5
leucine	5 / 5	2.0	5
lysine	2 / 5	0.25	5
methionine	2 / 5	1.0	5
ornithine	5 / 5	*	5
phenylalanine	5 / 5	2.5	5
proline	5 / 5	2.5	10**
serine	5 / 5	0.5	5
threonine	3 / 5	1.0	5
tryptophane	2.5/ 5	3.0	10**
tyrosine	1.5/ 5	3.0	15**
valine	5 / 5	2.0	5
asparagine	5 / 5	1.0	5

*Source of reference does not list minimum amounts detectable.

**Large concentrations are used since THESE AMINO AC'DS ~~proline and hydroxyproline~~ give a faint color ~~yellowish~~ reaction with ninhydrin in smaller concentrations.

Sheets of Whatman No. 1 filter paper, 42.5 cm. by 42.5 cm., were marked with a line drawn parallel to and 3.0 cm. from the bottom edge of the paper, and a mark is made 3.0 cm. from one edge along this line. This indicates the point of application of the sample. The samples are applied by means of a micropipette by gently touching the tip to the paper at the designated point; 5 µl were applied at one time. If more sample is required the original spot is allowed to dry first. In this manner 50 µl or more of the sample can be applied without increasing the area of the spot at the point of application. The papers were then hung in a hood and subjected to steam for 15 minutes. This process results in a more uniform chromatogram and very compact spots. The filter papers were then allowed to develop in the phenol tanks in the presence of ammonia vapor, for 36 hours. The papers were then removed and the phenol evaporated in the hood by a current of air. It is important that all traces of phenol be removed, otherwise the finished chromatogram may be damaged. The distance the solvent travels is then measured and each sheet was cut below the solvent front in order to remove a large part of the phenol decomposition products, recognized by the green band across the solvent front. The chromatograms were then steamed as described above and placed in a second tank to which butanol-acetic acid mixture was added. The chromatograms were allowed to develop for 16 hours and then the same process of drying was followed. The distance which this solvent travels is best seen under ultraviolet light.

The chromatograms were sprayed with a 0.2% ninhydrin solution and the reaction allowed to take place during 24 hours at room temperature. The distance each amino acid traveled in both solvents was measured and the R_f values determined. (The R_f value is defined as the ratio of the distance the solvent travels to the distance the spot travels.)

Specific methods of identification.

a. Identification by comparison of R_f values of known amino acids and those found in the mycelial hydrolysates. -- The R_f values of 22 known amino acids were determined separately on individual filter paper sheets. Samples of mycelium grown in the test culture mediums were hydrolyzed as per procedure and the amino acid residue made up to 5 ml. with 75% ethanol. Twenty µl. of sample were spotted, 5 µl at a time, and chromatograms were allowed to develop. By comparing the R_f values of the known amino acids with R_f values of amino acids from the test samples the identity of the latter are determined. However, further proof is necessary since certain spots, although separate from one another, appear close together and it is often difficult to base conclusions on R_f values alone.

b. Identification from the pattern of spots. -- Having located the position of the known amino acids by R_f values, as above, the next step involves spotting the paper with various mixtures of knowns, e.g., a mixture of 2, 4, 8, 10, etc. amino acids, by superimposing the individual amino acid chromatograms from the above on that of the mixtures. In this way individual amino acids in the mixture can be identified as to their position on the chromatogram and thus a pattern of spots is established for the 22 known acids.

BRIEF SUMMARY OF WORK TO DATE

Certain aspects of protein synthesis by the wood-rotting fungi are being studied. Polyporus palustris, a brown rot, was grown in quantity by the submerged culture technique, using three media each containing a different form of nitrogen. Growth from two chemically defined media -- glucose-mineral-ammonium nitrate and glucose-mineral-glutamic acid -- was compared with that from 2 per cent malt extract solution. The organism was grown in submerged culture for 10 days at 28° C., then the pellets of growth removed, washed and lyophilized to furnish material for analysis. A micro-Kjeldahl method was used for total nitrogen, and

paper chromatography for identifying amino acids in hydrolysates of the mycelium. The oven-dry weights of mycelium produced in the ammonium nitrate, glutamic acid and malt extract media were, respectively, 3.9, 4.8 and 5.6 grams, while the corresponding percentages of protein (N x 6.25) in the mycelium were 36.9, 33.1 and 29.4. The following amino acids were identified in mycelium from the three different culture media: a-alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine. Cystine, cysteine, hydroxyproline, ornithine and asparagine were not found. Although the <u>type</u> of nitrogen in the medium had some influence on the <u>amounts</u> of mycelium produced and of protein synthesized, it had no effect on the <u>kinds</u> of amino acids formed.

PROGRESS

<u>Results since January, 1953</u>

A. Nitrogen, protein and ash of <u>P. palustris</u> mycelium.

The organism was grown, harvested and preserved as indicated under Basic Procedures (A, B, and C), after a 10-day growth period. The test culture media were:

1. <u>Ammonium nitrate medium</u>.

   Basal synthetic medium with ammonium nitrate, 0.342 g./liter, as the nitrogen source. Total nitrogen = 120 mg./liter (0.012%).

2. <u>Glutamic acid medium</u>

   Basal synthetic medium with glutamic acid, 0.75 g./liter, as the nitrogen source. Total nitrogen = 120 mg./liter (0.012%).

3. <u>Malt extract medium</u>.

   2% malt extract (Difco, desiccated) in distilled water. Total nitrogen = 80 mg./liter (0.008%).

All media were adjusted to pH 5.5 before inoculation.

Analyses of the mycelium for total nitrogen and ash, and calculations for protein, were made as indicated under Basic Procedures (D), using growth from each of the three culture media. Table 1 shows the results.

TABLE 1

NITROGEN, PROTEIN AND ASH OF P. PALUSTRIS MYCELIUM FROM THREE CULTURE MEDIA. AVERAGES OF 8 DETERMINATIONS ON MYCELIUM FROM EACH MEDIUM. OVEN-DRY BASIS.

Medium	Grams dry weight of mycelium per 5 liters of medium	Percent nitrogen	Percent protein (N x 6.25)	Percent ash
2% malt extract	5.6	4.7	29.4	5.4
Glutamic acid	4.8	5.3	33.1	2.5
Ammonium nitrate	3.9	5.9	36.9	2.9

B. Amino acids of P. palustris mycelium.

Qualitative analyses of mycelial hydrolysates for amino acids were made as indicated under Basic Procedures (E), using growth from each of the three culture media. Acid hydrolysates were used for all amino acid determinations except tryptophane, which is destroyed by acid.

The results are based on comparison R_f values of known amino acids and of the unknown amino acid spots on chromatograms. Table 2 shows the results for mycelium from the three media. The R_f standards are included for 5 amino acids tested for but not found in the hydrolysates.

TABLE 2

AMINO ACIDS OF P. PALUSTRIS MYCELIUM FROM THREE CULTURE MEDIA. R_f VALUES OF UNKNOWNS AND OF STANDARDS COMPARED.

	R_f values								
Amino acid	Standards		NH_4NO_3 medium		Glutamic medium		Malt medium		
	Phenol	Butanol-acetic ac.	Phenol	Butanol-acetic ac.	Phenol	Butanol-acetic ac.	Phenol	Butanol-acetic ac.	Color-Ninhydrin
a-alanine	.60	.32	.60	.31	.62	.34	.63	.31	Purple
arginine	.86	.11	.89	.11	.84	.10	.86	.11	Purple
asparagine	.34	.10	0	0	0	0	0	0	Orange-brown
aspartic acid	.13	.17	.14	.22	.15	.17	.15	.19	Blue "
cysteine	.17	.03	0	0	0	0	0	0	Blue
cystine	.05	.09	0	0	0	0	0	0	Blue
glutamic acid	.26	.21	.29	.24	.25	.22	.28	.25	Blue "
glycine	.40	.19	.38	.17	.43	.19	.39	.20	Red "
histidine	.66	.11	.66	.10	.64	.10	.68	.13	Purple
hydroxyproline	.63	.24	0	0	0	0	0	0	Yellow
isoleucine	.87	.59	.86	.58	.87	.59	.87	.59	Purple
leucine	.88	.61	.87	.59	.88	.63	.88	.62	Purple
lysine	.73	.11	.73	.08	.74	.13	.72	.10	Purple
methionine	.79	.48	.78	.46	.82	.44	.79	.48	Dull "
ornithine	.62	.07	0	0	0	0	0	0	Purple
phenylalanine	.88	.53	.90	.54	.87	.54	.87	.50	Dull "
proline	.88	.32	.90	.34	.86	.30	.88	.31	Yellow
serine	.28	.18	.26	.17	.29	.17	.30	.19	Purple
threonine	.44	.24	.46	.25	.45	.22	.44	.24	Purple
tryptophane	.68	.32	.70	.33	.72	.36	.68	.36	Purple
tyrosine	.59	.38	.60	.36	.60	.40	.58	.37	Dull "
valine	.80	.47	.81	.51	.78	.48	.80	.51	Purple

CONCLUSIONS

P. palustris, grown in submerged culture in a synthetic ammonium nitrate medium, a synthetic glutamic acid medium and a 2 per cent malt extract solution, respectively, produced the least amount of mycelium in the first medium and the greatest amount in the last-named. The percentages of nitrogen (and protein) in the mycelium from the three media were roughly inversely proportional to the mycelial weights. Seventeen amino acids were identified in the mycelium from each medium, while five other amino acids were lacking in all cases. Although the type of nitrogen in the growth medium had some influence on the amounts of mycelium produced and of protein synthesized, it had no effect on the kinds of amino acids formed.

BIBLIOGRAPHY

No papers have been published during the period January 1,1953 to June 30,1953.

OTHER ASPECTS OF THE STUDIES

a. Changes in emphasis or orientation.

None.

b. Personnel changes.

None.

c. Graduate students on contract.

Messrs. Chester Koda and Maurice Fagan - each half-time.

d. Other research support.

The Department of Plant Sciences continues to furnish secretarial assistance, space and certain capital equipment, including a new micro-Kjeldahl apparatus.

e. Difficulties encountered

None.

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